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Mammalian ER stress sensor IRE1β specifically down-regulates the synthesis of secretory pathway proteins

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ABSTRACT

Accumulation of unfolded proteins in the endoplasmic reticulum (ER) causes ER stress. The ER stress sensor inositol requiring enzyme-1beta (IRE1β), which is specifically expressed in intestinal epithelial cells, is thought to be involved in translational repression. However, its mechanism of action is not fully understood. Using a reporter that can evaluate and distinguish between translation efficiency in the cytosol and on the ER membrane, we show here that IRE1β represses translation on the ER membrane but not in the cytosol, and that this selective repression depends on the RNase activity of IRE1β.

1. Introduction

Secretory and transmembrane proteins, as well as certain organelle-targeted proteins, are translated by endoplasmic reticulum (ER) membrane-bound ribosomes and translocated into the ER. Translocated proteins are folded, glycosylated and disulfide-bonded with the help of ER chaperones and enzymes. However, an excess influx of proteins can perturb these processes, resulting in ER stress. To protect themselves, cells then activate the unfolded protein response (UPR), a pathway which includes the transcriptional induction of ER chaperones, folding enzymes and components of ER associated degradation (ERAD), and the attenuation of general protein translation [1].

In mammals, IRE1, PERK and activating transcription factor 6 function as ER stress sensors. PERK and IRE1 are type I ER transmembrane proteins that include an ER lumenal sensor domain and a cytosolic effector domain. Under ER stress, PERK phosphorylates the translation initiation factor eukaryotic initiation factor 2α with its kinase to repress translation globally [2]. IRE1 has cytosolic kinase and autophosphorylation-dependent endoribonuclease (RNase) activities. Two IRE1 paralogues, IRE1α and IRE1β, have been reported in mammals [3,4]. IRE1α catalyzes the spliceosome-independent processing of the pre-mRNA that encodes transcription factor XBP1. This leads to the production of the mature form of XBP1 protein, which in turn induces UPR target genes [5,6]. Further ubiquitously expressed IRE1α is essential for placental development and embryonic viability [7]. On the other hand, a dispensable IRE1β is specifically expressed in epithelial cells of the gastrointestinal tract [8]. Although IRE1β has been reported to be competent to cleave *XBP1* mRNA [6], we found that its ability was less efficient than IRE1α [9]. In contrast, overexpression of IRE1β in HeLa cells induces 28S rRNA cleavage [10] more efficient than IRE1α [9]. Recently, it has been reported that IRE1α and IRE1β degrade ER-localized mRNA [11–16], but most findings were obtained from the analysis of IRE1α. In this study, we have constructed a useful reporter system for evaluation of translation efficiency between cytosolic and secretory protein synthetic pathways, and have found that hIRE1β selectively suppresses the synthesis of secretory protein translation by membrane-bound ribosomes through its own RNase activity.

Abbreviations: ER, endoplasmic reticulum; UPR, unfolded protein response; hIRE1, human inositol-requiring enzyme 1; hPERK, human PKR-like endoplasmic reticulum kinase; FBLN1, fibulin 1; B2M, beta-2-microglobulin; FBN1, fibrillin 1; CD59, CD59 molecule, complement regulatory protein; LAMP1, lysosomal-associated membrane protein 1; TFRC, transferrin receptor; SCARA3, scavenger receptor class A member 3; GYLTL1B, glycosyltransferase-like 1B; ACTB, actin beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NFKB1, nuclear factor kappa-B p105 subunit; TBP1, TATA box binding protein

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2. Materials and methods

2.1. Cell culture and transfection with plasmids

HeLa cells (HeLa Tet-Off, Clontech) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. The calcium phosphate-DNA precipitation method was used to introduce plasmid DNA into HeLa cells.

2.2. Luciferase assay

For translational assays, HeLa cells were seeded in six-well plates at 5×10^5 cells/well. After 18 h, the cells were transfected with 2.0 μg of a human PKR-like endoplasmic reticulum kinase (hPERK) or human inositol-requiring enzyme 1 (hIRE1) expression plasmid, 1.8 μg of pFL-SV40-ER and 0.2 μg of pRL-SV40. Translational efficiency was measured using the dual luciferase assay system (Promega) and luminescence was detected with a luminometer (AB-2200-R, ATTO).

2.3. Western blot analysis

After SDS–PAGE, the proteins were electrotransferred onto a nitrocellulose membrane. Proteins on the membrane were immunodetected with anti-luciferase pAb (Promega), anti-HA (12CA5) (Roche), or anti-GAPDH ab9484 (Abcam).

2.4. Real-time PCR

We purified RNA using RNAiso plus (Takara). After synthesis of cDNA from total RNA using SuperScript II (Invitrogen), we performed quantitative real-time PCR in duplicate with a LightCycler480 (Roche) and the primers shown in Table S1.

2.5. Additional methods

Methods for plasmid construction, fractionation of free and membrane-bound ribosomes and evaluation of translational activity are described in Supplementary methods.

3. Results

3.1. hIRE1β specifically inhibits the translation of a secretory pathway protein

It seems reasonable to suppose that protein translation in the cytosol should not be suppressed under ER stress, because essential cytosolic activities would otherwise be debilitated. Since hIRE1β-mediated translational repression is partial [10], we anticipated that hIRE1β might only repress translation on ER membranes. To evaluate the translational efficiencies of free and membrane-bound ribosomes separately, we established a modified dual luciferase assay system. Since native firefly and renilla luciferases are translated by free ribosomes (Fig. S1), we constructed a plasmid that expresses an ER-localized firefly luciferase bearing the calreticulin signal sequence and a KDEL ER retrieval signal (Fluc-ER) (Fig. 1A), which would be translated by membrane-bound ribosomes. Fluc-ER also contains a putative N-glycosylation site to serve as an indicator of its ER localization. In Fluc-ER-overexpressing cell lysates, we could detect two bands by Western blotting with anti-firefly luciferase antibody (Fig. 1B, lane 1). The major upper band declined in cells treated with tunicamycin (Tm), an inhibitor of N-linked glycosylation [17] (Fig. 1B, lane 2), and disappeared after endoglycosidase H (Endo H) treatment (Fig. 1B, lane 4). We confirmed that the unglycosylated form of Fluc-ER also translocated into the ER (Fig. S1). These results show that Fluc-ER localizes in the ER. We also used renilla luciferase (Rluc-Cyt) as a cytosolic translation marker to compare translation efficiencies in the cytosol and on the ER membrane within the same cell.

Using this assay system, we observed the effects of overexpression of ER stress sensors on the translation of Fluc-ER and Rluc-Cyt, since these ER stress sensors are activated by their own overexpression [11]. When hPERK was overexpressed, translation of both Fluc-ER and Rluc-Cyt was inhibited, but the ratio of Fluc-ER/Rluc-Cyt expression did not change significantly (Fig. 1C). This is consistent with a report that PERK attenuates general translation [2]. In the case of hIRE1β overexpression, only the translational efficiency of Fluc-ER was dramatically decreased, and the Fluc-ER/Rluc-Cyt expression ratio dropped to less than 0.2. hIRE1α overexpression stimulated the activity of Rluc-Cyt, but had little effect on that of Fluc-ER. We confirmed that the activity of Fluc-ER correlated with its protein level, and that HA-tagged stress sensors were highly expressed (Fig. 1D). These data indicate that hIRE1β suppresses the translation efficiency of only membrane-bound ribosomes.

3.2. Reduction of ER-localized mRNA levels is mediated by the RNase activity of hIRE1β

To determine which domain in hIRE1β is required for selective translational suppression, we co-expressed the hIRE1 mutants shown in Fig. 2A with Fluc-ER and Rluc-Cyt. hIRE1αK599A (αK599A) and hIRE1βK547A (βK547A) are kinase-dead mutants that lack both kinase and RNase activity, whereas αK907A and βK855A are RNase-dead mutants that retain kinase activity but lack RNase activity [11]. βΔR is an RNase domain-deleted mutant. Selective suppression of Fluc-ER activity was detected in cells overexpressing hIRE1β wild type (WT), but not in those overexpressing βK547A, βK855A or βΔR (Fig. 2A). Interestingly, a chimeric mutant β/αR, in which the hIRE1β RNase domain (777–925 a.a.) was replaced by that of hIRE1α (829–977 a.a.), did not reduce the Fluc-ER/Rluc-Cyt ratio. It should be noticed that β/αR chimera has an ability to splice *XBP1* mRNA in vivo like IRE1α in our previous work [9]. In contrast, α/βR, in which the hIRE1α RNase domain was replaced by that of hIRE1β, did reduce the value of Fluc-ER/Rluc-Cyt compared with αWT. These results indicate that the RNase activity of hIRE1β is important for the selective suppression of Fluc-ER translation.

Is this hIRE1β-mediated selective suppression caused by the cleavage of 28S rRNA? To answer this question, we compared the amount of cleaved 28S rRNA in free and membrane-bound ribosomes. In cells overexpressing hIRE1β WT, 3′-fragment of 28S rRNA appeared not only in the membrane-bound ribosome fraction but also in the free ribosome fraction (Fig. 2B). If ribosomes containing cleaved 28S rRNA would display reduced translation efficiency, translation by free ribosomes, as well as that by membrane-bound ribosomes, should decrease. However, this is not consistent with the selective suppression of Fluc-ER by overexpression of hIRE1β, suggesting that ribosomes harboring cleaved 28S rRNA have little effect on normal translational efficiency in this assay system.

We also examined the effects of ER stress sensors on reporter mRNA by real-time PCR. Reduction of *Fluc-ER* mRNA was clearly observed in cells overexpressing hIRE1βWT or hIRE1α/βR (Fig. 2C). These results support the idea that the RNase activity of hIRE1β reduces *Fluc-ER* mRNA. However we failed to observe such a drastic effect in cells overexpressing IRE1α in this experiment.

3.3. hIRE1β down regulates endogenous ER-localized mRNA to limit the influx of newly synthesized proteins into the ER

We used metabolic labeling to examine whether hIRE1β attenuates the translation of endogenous ER proteins. After label-

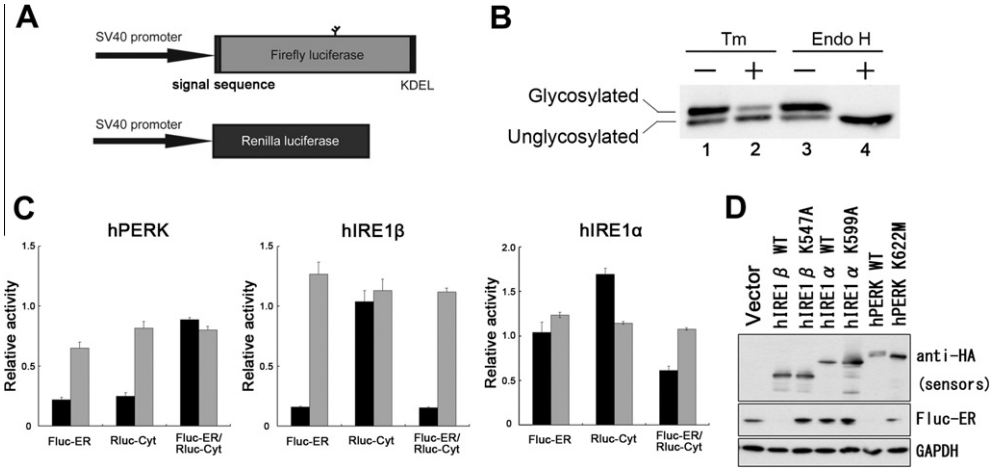

Fig. 1. Specific down-regulation of ER-localized firefly luciferase by hIRE1β. (A) Reporters used to detect the efficiencies of translation on the ER membrane (Fluc-ER, upper) and in the cytosol (Rluc-Cyt, lower). Fluc-ER contains the calreticulin signal sequence and KDEL at the N and C termini of firefly luciferase, respectively. Fluc-ER has one putative N-linked glycosylation site (Asn at residue 197 of native firefly luciferase). (B) N-linked glycosylation of Fluc-ER was examined by Western blotting. After transfection with *Fluc-ER*, HeLa cells were incubated for 12 h with or without 0.5 μg/ml of tunicamycin (Tm) (lanes 2 and 1), then lysed, and the lysate was treated with endoglycosidase H (Endo H) (lane 4). Upper and lower bands are the glycosylated and unglycosylated forms of Fluc-ER, respectively. (C) HA-tagged hPERK-WT, hPERK-K622M, hIRE1αWT, hIRE1αK599A, hIRE1βWT or hIRE1βK547A were overexpressed together with Fluc-ER and Rluc-Cyt into HeLa cells. Each mutant is kinase-deficient. At 24 h after transfection, cells were lysed and dual luciferase assays were performed. The graphs indicate the relative activities of Fluc-ER, Rluc-Cyt, and Fluc-ER/Rluc-Cyt. The luciferase activity in cells transfected with Fluc-ER- and Rluc-Cyt-expressing plasmid together with control vector was set to 1.0. Black bars indicate wild type stress sensors and gray bars indicate kinase deficient mutant of stress sensors. Error bars indicate the S.D. of triplicate experiments. (D) To assess the expression levels of each HA-tagged ER stress sensor and of Fluc-ER, Western blot analysis was performed using the same lysates described in (C). hIRE1β-HA, hIRE1α-HA, and hPERK-HA were detected with anti-HA antibody, and Fluc-ER was detected with anti-firefly luciferase antibody. As an internal control, GAPDH was detected with anti-GAPDH antibody. WT, wild type; KM, kinase-deficient mutant.

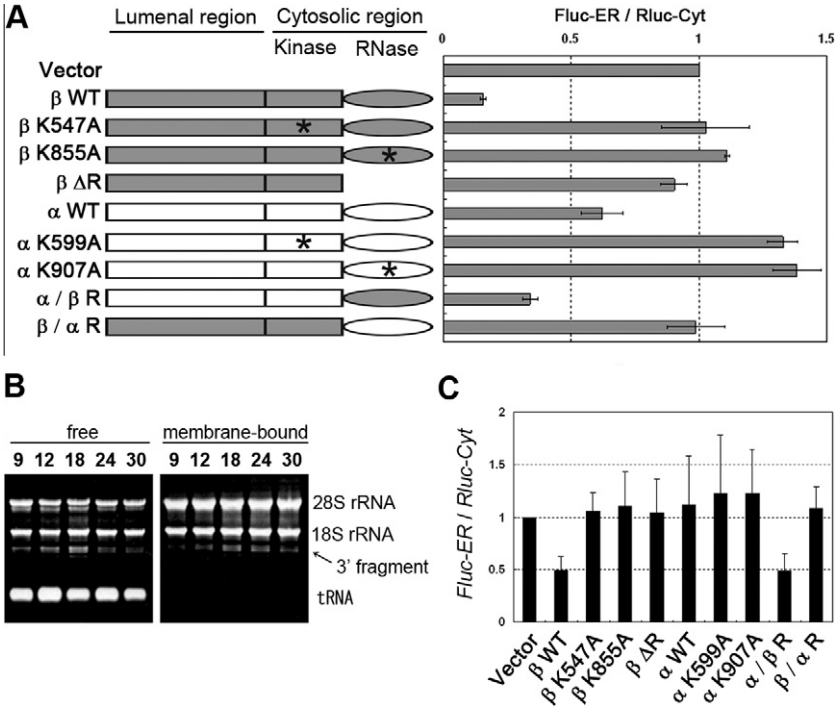

Fig. 2. hIRE1β reduces *Fluc-ER* mRNA levels via its RNase activity. (A) The indicated hIRE1 variants were transfected together with Fluc-ER and Rluc-Cyt into HeLa cells. At 24 h after transfection, cells were lysed and dual luciferase assays were performed. The graph on the right indicates the values of Fluc-ER/Rluc-Cyt ratios; all values were normalized to vector control. To the left of the graph, schematic representations of the transfected hIRE1s and their mutants are also shown. αWT, αK599A, αK907A, βWT, βK547A, βK855A, βΔR, α/βR and β/αR are described in the text. * indicates the mutated position. The luciferase activity in cells transfected with Fluc-ER and Rluc-Cyt-expressing plasmid together with control vector was set to 1.0. Error bars indicate the S.D. of triplicate experiments. (B) hIRE1β-overexpressing cells were permeabilized by digitonin, and cytosolic RNAs were extracted from this soluble material (free ribosome fraction). From the remaining material, membrane-bound ribosomes were extracted (membrane-bound ribosome fraction). Each fraction of total RNA was separated on 1% denaturing agarose gels and stained with ethidium bromide. (C) The hIRE1 variants shown in (A), Fluc-ER and Rluc-Cyt were cotransfected into HeLa cells. At 24 h after transfection, RNA was isolated and the relative amounts of mRNA were quantified by real-time PCR. *Fluc-ER* mRNA levels were normalized to those of *Rluc-Cyt* mRNA and to vector controls. Error bars indicate the S.D. of quadruplicate experiments.

ing with [^{35}S]methionine/cysteine, cells were lysed and fractionated into cytosolic proteins and N-linked glycoproteins that have binding affinity to ConA (concanavalin A). Measurements of isotope incorporation into TCA-insoluble material for each sample showed that glycoprotein synthesis was considerably lower than cytosolic protein synthesis in hIRE1βWT-transfected cells,

whereas protein synthesis in the two fractions was comparable in both hIRE1βK547A- and vector-transfected cells (Fig. 3A). Analysis of each fraction by SDS–PAGE showed that a wide range of glycoproteins were decreased in hIRE1β WT-overexpressing cells (Fig. 3B), suggesting that hIRE1β is a general inhibitor of translation on ER membranes.

To substantiate these data, the levels of selected endogenous cytosol- and ER-localized mRNAs were measured by real-time

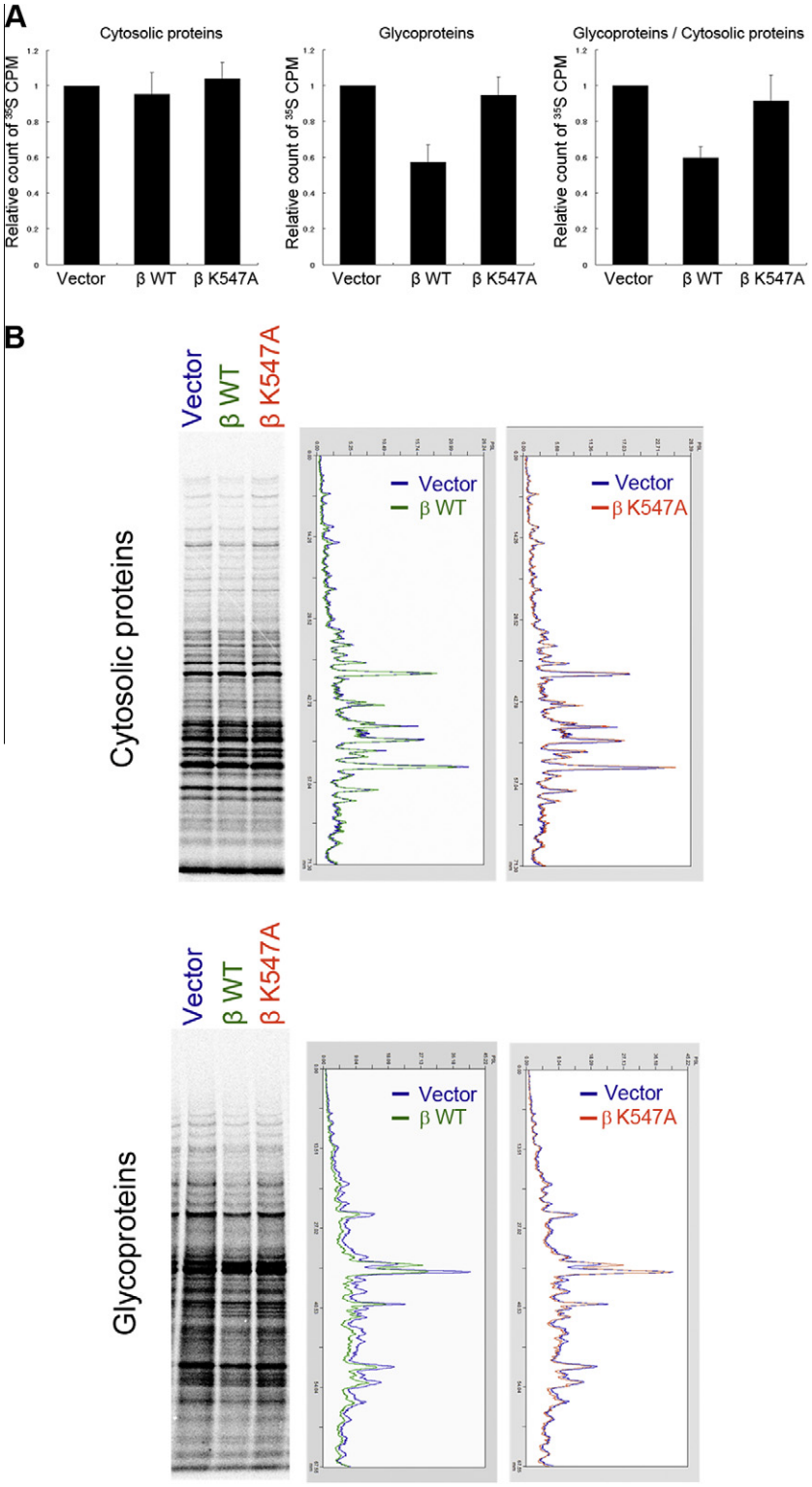

Fig. 3. hIRE1β inhibits the synthesis of endogenous glycoproteins. (A) hIRE1βWT- and hIRE1βK547A-overexpressing HeLa cells were labeled for 30 min with [^{35}S]methionine/cysteine before lysis. The lysates were fractionated into cytosolic proteins and glycoproteins. ^{35}S radioactivity in TCA-insoluble material from each fraction was measured. The three graphs show the radioactivity of ^{35}S (CPM) in cytosolic proteins and glycoproteins, and the ratio of radioactivity in glycoproteins to that in cytosolic proteins. All values were normalized to vector control. Error bars indicate the S.D. of quadruplicate experiments. (B) Samples of each fraction in (A) were separated by 2–15% SDS–PAGE and visualized by autoradiography. The density of each band in the autoradiographs was determined with Multi Gauge (Fujifilm). Blue trace indicates vector control, green indicates hIRE1βWT, and orange indicates hIRE1βK547A.

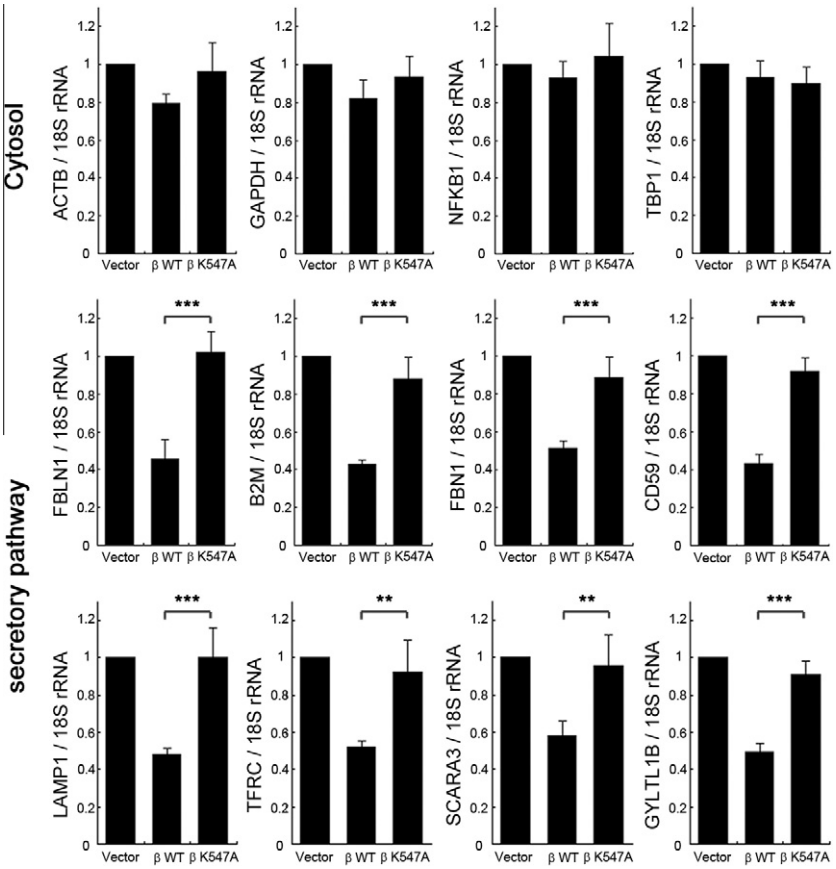

Fig. 4. hIRE1β suppresses endogenous mRNAs encoding secretory pathway proteins. Relative amounts of mRNA were quantified by real-time PCR. The top four graphs indicate the relative amounts of mRNAs encoding cytosolic (ACTB and GAPDH) or nuclear (NFKB1 and TBP1) proteins. The bottom eight graphs indicate the relative amounts of mRNAs encoding secretory proteins (FBLN1, B2M, and FBN1) and membrane proteins (CD59, LAMP1, TFRC, SCARA3 and GYLTL1B). RNA levels were normalized to those of 18S rRNA and to vector controls. Error bars indicate the S.D. of quadruplicate experiments. Asterisks indicate a significant difference from cells overexpressing hIRE1βK547A (**$P < 0.01$; ***$P < 0.005$) (Student's *t*-test).

PCR. hIRE1β suppressed all three mRNAs encoding secretory proteins [fibulin 1 (FBLN1), beta-2-microglobulin (B2M), and fibrillin 1 (FBN1)] and all five mRNAs encoding membrane proteins [CD59 molecule, complement regulatory protein (CD59), lysosomal-associated membrane protein 1 (LAMP1), transferrin receptor (TFRC), scavenger receptor class A member 3 (SCARA3) and glycosyltransferase-like 1B (GYLTL1B)]. In contrast, the levels of all four cytosolic mRNAs tested [actin beta (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), nuclear factor kappa-B p105 subunit (NFKB1) and TATA box binding protein (TBP1)] remained unchanged (Fig. 4A). These results indicate that hIRE1β acts as a global inhibitor of translation at the ER membranes.

4. Discussion

First we have established a useful dual reporter system that can simply evaluate and clearly distinguish between translation efficiency in the cytosol and on the ER membrane. Using this novel reporter system, we studied the function of IRE1β in HeLa cells. Several lines of evidence from our experiments suggest that IRE1β degrades mRNAs of secretory pathway proteins to prevent excess influx of newly synthesized proteins into the ER. Firstly, the mRNA level of firefly luciferase containing a signal sequence decreased remarkably in cells overexpressing hIRE1β WT, but hIRE1β did not affect the mRNA of cytosol-localized renilla luciferase (Fig. 2C). Secondly, the RNase domain and RNase activity of hIRE1β were important for the suppression of *Fluc-ER* mRNA (Fig. 2C). Thirdly, in cells overexpressing hIRE1β, the synthesis of glycoproteins translocated into the ER was attenuated (Fig. 3). Finally, the levels of several mRNAs encoding secretory pathway proteins were decreased in cells overexpressing hIRE1β (Fig. 4).

Although it has been reported that IRE1α and IRE1β are involved in the degradation of ER-localized mRNA [11–16], is there any difference in substrate between IRE1α and IRE1β? In our study, hIRE1β decreased the ER-localized *Fluc-ER* mRNA while hIRE1α had little effect on *Fluc-ER* mRNA (Fig. 2C). We also reported that hIRE1α efficiently cleaved specific sites of premature *XBP1* mRNA to facilitate the production of mature *XBP1* mRNA, but that hIRE1β barely did so [9]. We speculate that IRE1β as an RNase could have acquired evolutionarily substrate specificity that is slightly different from that of IRE1α, because IRE1β is specifically expressed in intestinal epithelial cells that also highly express IRE1α. IRE1α is known to cleave various mRNAs under strong ER stress by regulated Ire1-dependent decay (RIDD) [13], but its basic function may be to cleave precursor form of *XBP1* mRNA under physiological condition. As mentioned above, IRE1β exhibited lower activity in *XBP1* mRNA cleavage than IRE1α, and this study indicated that overexpression of IRE1β specifically reduced all nine ER-localized mRNAs (*Fluc-ER* mRNA and eight endogenous mRNAs) we examined without reduction of cytosolic mRNAs (Fig. 2C and Fig. 4). Thus RNase of IRE1β may have evolved to acquire a broader substrate specificity to regulate ER-localized mRNA stability and maintain ER homeostasis of highly differentiated secretory cells in the gastrointestinal tract under physiological condition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.12.002.

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